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Original research article

Gene Cloning and Protein Expression of Koi Herpesvirus ORF25

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ABSTRACT

Koi herpesvirus (KHV) caused significant morbidity and mortality in koi and common carp (*Cyprinus carpio*). Glycoprotein has been used for vaccine development as sub unit vaccine against many viruses. *KHV ORF25* is one of koi herpesvirus genes which encode a glycoprotein. The objectives of this research are to clone gene *KHV ORF25* and express its protein. The common carp showing necrosis and white patches of gill which was collected from Magelang was used in this research. Primers were designed to amplify partial ORF25 based on KHV J strain. *KHV ORF25* was successfully amplified and cloned in pET32a. Sequence analysis showed that this *KHV ORF25* has 99% homology with the sequences of KHV genotype KHV-J, KHV-I, and KHV-U. This ORF was predicted has 3, 23, and 8 B-cell epitopes based on Emini scale, Karplus and Schulz scale, and ElliPro respectively. The KHV ORF25 recombinant protein has been successfully produced in *Escherichia coli* as an insoluble protein with approximately 45 kDa in size. The high protein production was achieved when the protein induction was done at bacterial density at OD_{600} as 1.0 with 1-mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated at 37°C for 18 hours. The protein predicted has immunogenicity and the potency as a vaccine is needed to be evaluated.

1. Introduction

Diseases are major aquaculture problems that impact the quantity and quality of aquaculture products and significantly decrease the economy of aquaculture industry. Koi herpesvirus (KHV) also known as, cyprinid herpesvirus 3, koi herpes-like virus, and carp interstitial nephritis gill necrosis virus caused significant morbidity and mortality both in common carp and koi carp (Hedrick et al. 2000; Michel et al. 2010; Ilouze et al. 2011). The first outbreak of KHV was reported in 1998 and confirmed in 1999 in Israel. Since then, other cases have been confirmed in United States, Europe, and Asia (Hedrick et al. 2000). In Indonesia, KHV infection was started in Blitar, East Java, in March 2002. It spread rapidly through Java Island and caused very high mortality (80%–90%). From Java island the KHV spread into Sumatra, Borneo and other islands (Sunarto and Cameron 2006).

KHV is classified within the *Alloherpesviridae* family, consisting of a linear, double-stranded DNA genome of about 295 kbp packaged within an icosahedral capsid of characteristic architecture that is surrounded by a proteinaceous tegument layer and finally by a host-derived envelope containing virus glycoproteins (Aoki et al. 2007). Beside the linear form, Fujioka et al. (2015) also reported the genome KHV presence in circular or concatemeric double DNA form. This difference was suspected to be because KHV genome may be circularized for long-term maintenance without active viral replication. Aoki et al. (2007) reported that the complete genome sequences of KHV isolates from the USA (KHV-U), Israel (KHV-I) and Japan (KHV-J) share >99% nucleotide sequence identity. They suggested that an ancestral KHV initially diverged into two genetic lineages, KHV-U/I (European genotype) and KHV-J (Asian genotype). The polymerase chain reaction (PCR) patterns and sequence analysis based on the alleles of three domains of an alternate KHV classification system confirmed that the genotype of the KHV BA-08 isolate was cyprinid herpesvirus 3–third genotype (Kim and Kwon 2013)

Based on the observed pattern of polymorphism, Kurita et al. (2009) reported KHV samples were roughly divided into two groups, designated as Asian and European genotypes with each genotype containing two and seven variants, respectively. The homogeneity of Asian KHVs suggests that invasion of KHV into these areas occurred more recently and the virus spread rapidly when compared to the case of European KHV. The all 10 samples from

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Asia (Japan, Taiwan, Philippines, Indonesia) were classified as Asian Genotype with A1 and A2 variants. Subsequently, Dong et al. (2013) confirmed the emergence of the U/I genotype in adult koi suffering mass mortality in China in 2011. Based on the *TK* and *ORF136H* genes, the sequence analyses revealed that KHV-GZ11 is a distinct European genotype of KHV. This virus was further studied at a whole genome level by Li et al. (2015b).

Vaccination of fish is one way to prevent fish disease due to KHV infection. Yuasa and Sano (2009) reported that development of vaccine is one of the development of preventive measures against KHV in Japan. Immunologic studies by naturally resistant and immunization of attenuated virus have shown a significant elevation of the humoral response to vaccination. Furthermore, both viral inoculation and challenge showed very high anti-viral resistance and low mortality (Ronen et al. 2003). Adkison et al. (2005) showed that survivor of KHV outbreak showed high titer antibody against KHV and the passive immunization could protect carp from KHV infection. Yasumoto et al. (2006) and Miyazaki et al. (2008) have proved the efficacy of oral immunization with the liposome-KHV vaccine was efficacious against KHV infection in carp.

KHV ORF25, one of membrane glycoprotein of KHV (Aoki et al. 2007), has immunogenic properties as shown by DNA vaccination against KHV (Nuryati et al. 2010; Nuswantoro et al. 2012; Zhou et al. 2014). Beside the DNA vaccine, the recombinant proteins can be used as fish vaccines (Lecocq-Xhonneux et al. 1994; Sommerset et al. 2005; Lin et al. 2007; Shin et al. 2013; Kim et al. 2015; Li et al. 2015a), In this study we cloned KHV ORF25 from Indonesian virus, expressed the protein in *Escherichia coli* and explored the potency of it as a vaccine candidate.

2. Materials and Methods

2.1. Sample

Common carp (*Cyprinus carpio*) showing symptoms as necrosis and white patches of gills which was obtained from Magelang, Central Java in June 2012 was used in this study. DNA isolation was done from the gills using Tris-NaCl-EDTA-SDS (TNES) buffer method (Murwantoko 2009). Confirmation of KHV infection was carried out by PCR based on thymidine kinase primers, KHV-TKf (GGGTTACCTGTACGAG) and KHV-TKr (CACCCAGTAGATTATGC) (Bercovier et al. 2005).

2.2. Primer design

Previous studies showed that KHV Indonesian isolates were closely related to KHV-J from Japan (Murwantoko 2009; Sunarto et al. 2011; Murwantoko et al. 2012), so the primer sequences were designed based on TUMST1 KHV strain (Japan; accession number AP008984) as a template. The GC content, self-annealing loops, and hairpin loops were checked using Oligocalculator software (http://www.basic.northwestern.Edu/biotools/oligocalc.Html). Primers were designed to amplify DNA fragment which encoded partial ORF25 protein excluding the hydrophobic amino acids cluster. The primers were 28 bp in length with an additional *Eco*RI restriction site on the forward primer and *Hind*III restriction site in the reverse primer. Those primers are: KHV-ORF25-F: AGCTGAATTCGGTTCGAGGACCAACGTC, and KHV-ORF25-R: AGATAAGCTTCAGGTTTCCCCTGCGCCA.

2.3. DNA amplification and purifying from gel agarose

Amplification of DNA KHV using thymidine kinase and ORF25 primer pairs was performed using *Thermalcycler Mastercycler Personal* (Eppendorf, Germany) using PCR Mix Kappa (Biosystems). The PCR reaction was conducted as follows: one cycle of 95°C for 5 minutes, 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 75 seconds, followed by one cycle with 72°C for 5 minutes. PCR reactions were evaluated by 1% agarose electrophoresis (Nacalai) on Tris-acetate-ethylenediaminetetraacetic acid (TAE) (40 mM Tris-acetate pH 8.3, 1 mM ethylenediaminetetraacetic acid (EDTA)) using Mupid Advance horizontal electrophoresis (Advance, Japan). The gel was stained with ethidium bromide and observed under UV transilluminator (Extra Gene, Taiwan)

The PCR products of ORF25 were purified using phenol-chloroform and followed by digestion with *Eco*R1 and *Hind*III (Toyobo) at 37°C for 2 hours, then were agarose electrophoreted. The desired band DNA was excised and DNA was recovered from agarose using Ron's Gel Extraction kit (BIORON). Purified PCR fragment was ligated into pET-32a(+) (Novagen) using T4 DNA ligase (Toyobo) at 16°C overnight. Ligation mixture was transformed into *Escherichia coli* DH5α using heat shock on 42°C for 90 seconds followed by incubation on ice (Sambrook and Russell 2001). The bacteria were cultured on LB agar containing 50 μg/mL ampicillin overnight. The growth colonies were cultured in LB broth containing ampicillin and cultured at 37°C overnight. The plasmids were isolated from the bacteria using mini preparation of alkali lysis methods (Sambrook and Russel 2001). The presence of recombinant plasmid was checked by digestion of plasmid using *Eco*RI and *Hind*III enzymes (Toyobo).

2.4. Sequencing and data analysis

The recombinant plasmids were sequenced using S-Tag and T7-terminator primers by company service (Macrogen). DNA sequences from those two primers were overlapped to determine complete sequences. DNA sequences were analyzed using basic local alignment search tool (BLAST) to looking for the homology with the data on GenBank. The T-cell epitopes were predicted by GENETYX based on the pattern IAd (Sette et al. 1989) and patterns of Rothbard/Taylor (Rothbard and Taylor 1988). The prediction of B-cell epitope was conducted using the B Cell epitope Prediction Tools from immune epitope database (IEDB) (http://tools.iedb.org/main/html/bcell_tools.html) based on Emini surface accessibility scale (Emini et al. 1985), Karplus and Schulz flexibility scale (Karplus and Schulz 1985) and ElliPro (Ponomarenko et al. 2008).

2.5. Protein production

The recombinant plasmid of pET32-KHV-ORF25 was transformed into *E. coli* BL21 DE3 using heat shock method described previously. Bacteria were inoculated into 100 mL of LB broth containing ampicillin (50 μg/mL), then incubated with shaker at 37°C overnight. The bacterial culture was inoculated into 1000 mL LB broth then the protein production was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) into medium. Bacterial cells were harvested by centrifugation at the speed of 3500 rpm for 15 minutes on *Refrigerated Centrifuge 5810R* (Eppendorf). Bacterial pellet was collected and resuspended in 10% of the medium volume of phosphate-buffered saline. Bacterial cells were disrupted by sonication using Ultrasonic Generator US-300T (Nissei, Japan).

The solubility of protein was checked by centrifugation of sonicated bacterial suspension at 10,000 *g* for 3 minutes to separate soluble and insoluble fractions. The optimization of protein production was performed by combination of different bacterial density, concentration of IPTG and length of induction. Those treatments were induction at optical densities (OD 600) 0.6, 0.8, and 1; induction at IPTG concentrations 0.6, 0.8 and 1 mM; and length of induction 3, 6, and 18 hours. The protein was analyzed using 10% SDS-PAGE (Sambrook and Russel 2001) using PAGE run vertical electrophoresis AE-6530 (Atto, Japan) stained by Coomassie brilliant blue.

2.6. Protein purification

Protein purification from sonicated bacterial suspension was done using Ni-NTA agarose (Qiagen) in accordance with the

protocol of QIAexpressionist kit on denatured conditions using washing buffer (100 mM NaH_2PO_4, 10 mM TrisHCl, 8 M urea) pH 8, 6.3, 5.9 and elution buffer (100 mM NaH_2PO_4, 10 mM TrisHCl, 8M urea) pH 4.5. The protein was analyzed using SDS PAGE 12%.

3. Results

3.1. KHV ORF25 amplification

The common carp sample from Magelang was confirmed to be infected by KHV. PCR analysis with KHV-TKf and KHV-TKr primers (Bercovier et al. 2005) using DNA from gill tissue as template produced single band at approximately 400 bp (Figure 1A lane 1). From that DNA sample, the membrane protein ORF25 was amplified using KHV-ORF25-F/KHV-ORF25-R primers. The single band in approximately 1000 bp size was produced at expected size (Figure 1B lane 1). The PCR product was digested using *Eco*RI and *Hind*III (Figure 1B lane 2) and used for cloning.

3.2. Cloning

Transformation of ligation mixtures of pET32 and DNA insert into *E. coli* DH5α produced four colonies in LB agar containing ampicillin. Evaluation of the presence of recombinant by agarose electrophoresis showed that a plasmid has higher molecular weight (Figure 2A lane 1) compared to the other plasmids (Figure 2A lane 2). Plasmid digestion by *Eco*RI and *Hind*III restriction enzymes reconfirmed that the plasmid was a recombinant plasmid as a 1000 bp band appeared (Figure 2B lane 1), whereas for the empty plasmid, the band did not appear (Figure 2B lane 1). The size of the inserted DNA was the same with the PCR product using KHV-ORF25-F/-KHV-ORF25-R primers (Figure 2B lane 3).

3.3. Sequencing and data analysis

The recombinant plasmid was sequenced using S-Tag and T7-terminator primers. The sequences from those primers were overlapped to determine sequence of cloned *KHV ORF25* gene. The size of full length ORF25 are 1833 nt on KHV-TUMST1 strain and 1805 nt on KHV-U and KHV-I strains. Sequencing results showed that this *KHV ORF25* was composed only of 1012 nt, it means that this *KHV ORF25* is only partial gene (Figure 3). This sequence has been submitted to GenBank with accession no KX538957. BLAST

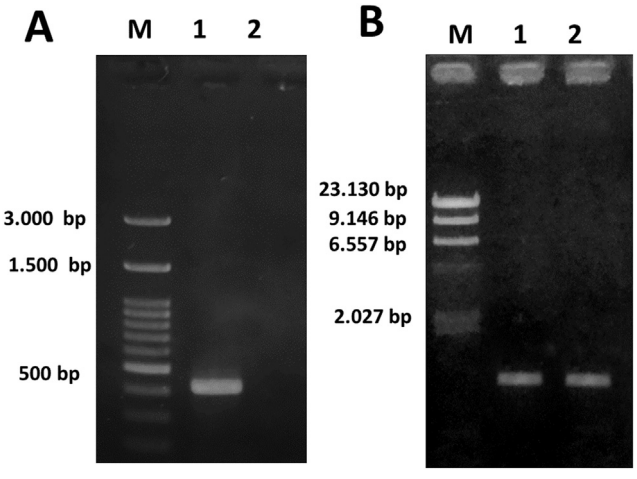

Figure 1. Agarose gel electrophoresis of KHV PCR products of confirmation of presence of KHV with Bercovier primer (A) M. 100 bp DNA ladder. 1. DNA sample as template. 2. Negative control. Agarose gel electrophoresis of KHV PCR products of ORF25 (B) M: λ/*Hind*III DNA marker, 1: KHV ORF25 amplicon, 2: Digested KHV ORF25 amplicon with restriction enzymes.

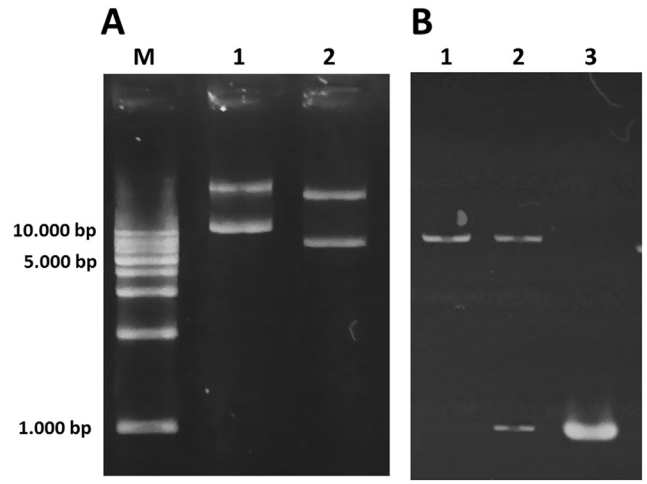

Figure 2. Agarose electrophoresis of isolated plasmid at native condition (A) and at digested with *Eco*RI and *Hind*III enzymes (B). (A) M: 1 KB DNA ladder, lane 1: recombinant plasmid, lane 2: empty plasmid. (B) Lane 1: empty plasmid, lane 2: recombinant plasmid, lane 3: KHVORF25 amplicon.

analysis showed that only nine data entries were showing significant high homology. The result showed 99% homology with KHV genotype KHV-J (Japan), KHV-I (Israel), and KHV-U (USA) (Aoki et al. 2007), strain KHV-GZ11 (China) (Li et al. 2015b), isolate SNUKHV (South Korea), strains FL (Belgium), isolate HZ419 (China), isolate FL (Belgium). Translation into amino acids was conducted using GENETYX program as presented in Figure 3. The multiple alignment of this KHV with KHV strains from GenBank showed there is one nucleotide different at number 254 (cytosine from this isolate instead of thymine in other KHV strains) and produced different nucleotide at translation level as alanine instead of valine (data not shown).

Prediction of T-cell epitope of this KHVORF25 using the GENETYX program shows nine epitopes recognized by IAd pattern and eight epitopes recognized by Rothbard/Taylor pattern. One epitope is recognized by both IAd and Rothbard/Taylor pattern. The prediction of B-cell epitope of KHV ORF25 was conducted using the B Cell epitope Prediction Tools. The analysis based on Emini surface accessibility scale, Karplus and Schulz flexibility scale and ElliPro predicted KHV ORF25 contains 3, 23, and 8 epitopes, respectively. At least three epitopes were predicted from two scales and two epitopes were recognized by all three scales (Figure 3).

3.4. Protein production

The protein production was conducted under pET32a expression system. Because *KHV ORF25* was a partial gene, the start codon and stop codon come from plasmid. KHV ORF25 protein was successfully produced in *E. coli* BL21. It can be seen on the presence of protein at approximately 45 kDa size as a dominant band in SDS PAGE. The protein expression can be induced by addition of IPTG at bacterial density at OD 0.6, 0.8, and 1.0. The protein production increases following the incubation hours of 3, 6 and 18 hours. From those conditions, we can see that induction at bacterial density at OD 1.0 and induction time of 18 hours produce highest protein (Figure 4). IPTG induction at various concentrations of IPTG as 0.6, 0.8, and 1 mM did not show significant differences on protein production. All treatment showed the same protein band thickness (Figure 5A). Solubility test showed that recombinant protein ORF25 was expressed dominantly at insoluble condition (Figure 5B).

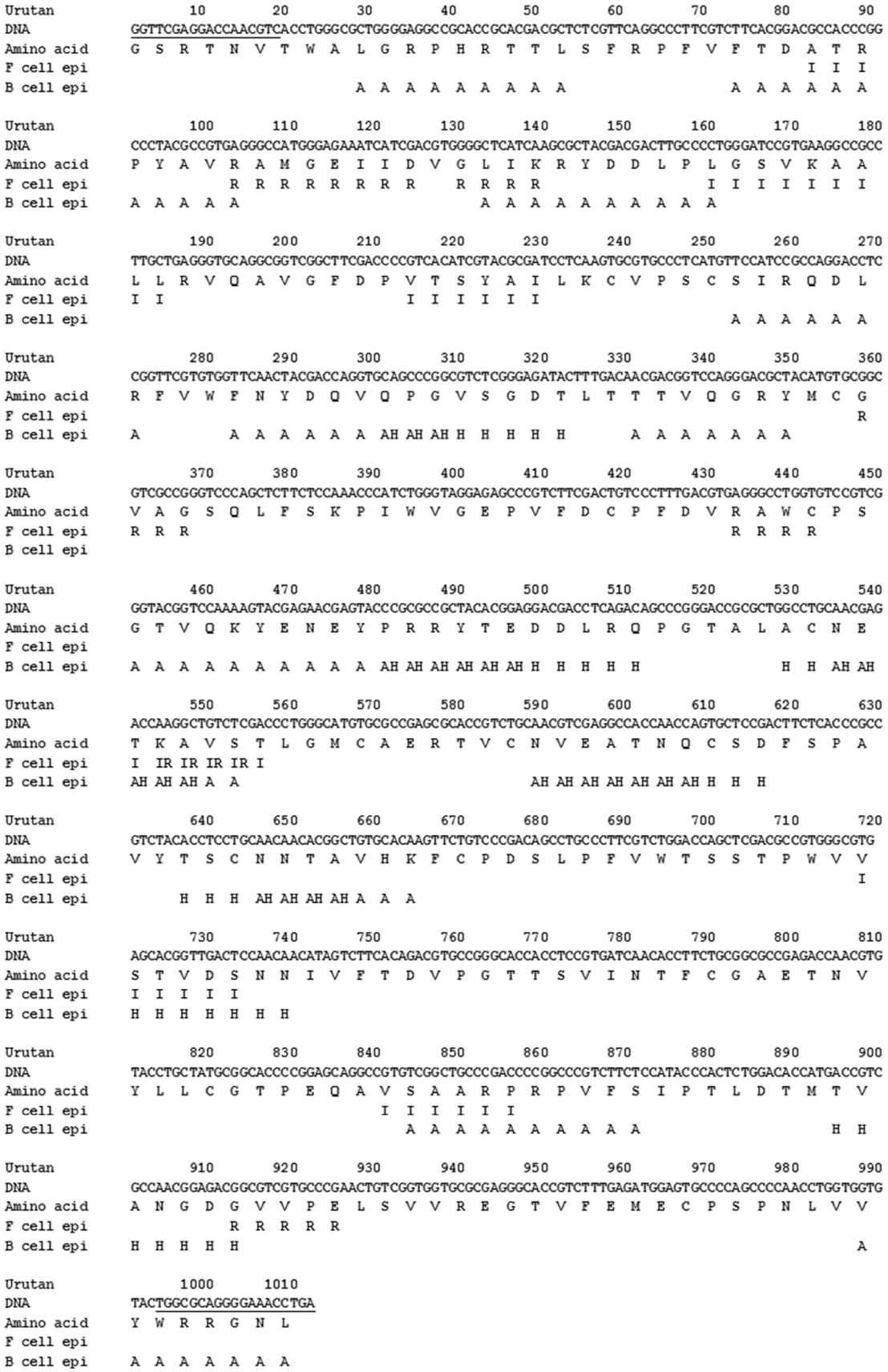

Figure 3. Nucleotide and amino acid sequences of KHV ORF25. Sequences with underlines indicate sequences of primers. The amino acids which were predicted as F-cell epitope based on IAd and Rothbard/Taylor patterns are indicated by I and R, respectively. The amino acids which were predicted as B-cell epitope based on Karplus and Schulz flexibility scale, Emini surface accessibility scale, and ElliPro are indicated by K, L and E, respectively.

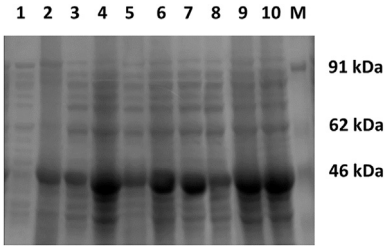

Figure 4. SDS PAGE of optimization of protein production by induction with 1 mM IPTG at different bacterial density and induction time. Lane 1: non-induction, lane 2–4: induction at density OD 0.6 with induction time for 3 (lane 2), 6 (lane 3), 18 hours (lane 4). Lane 5–7: induction at density OD 0.8 with induction time for 3 (lane 5), 6 (lane 6), 18 hours (lane 7), lane 8–10: induction at density OD 1.0 with induction time for 3 (lane 8), 6 (lane 9), 18 hours (lane 10). M: protein size marker with indicated size.

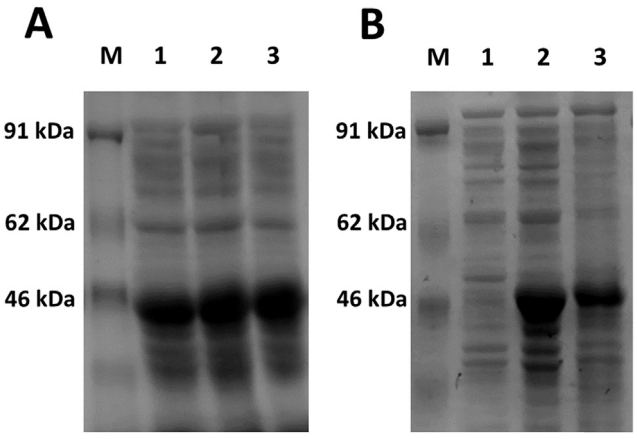

Figure 5. SDS PAGE of IPTG induction and solubility. Optimization of IPTG concentration for induction (A). *E. coli* at density OD 1.0 were induced with IPTG at 0.6 (lane 1), 0.8 (lane 2), and 1.0 mM (lane 3). M: Protein size marker with indicated size. (B) The sonicated bacterial suspension (A, lane 3) was centrifuged at 10,000*g* for 3 minutes; the supernatant collected as soluble fraction (lane 1); pellet was suspended in PBS as insoluble fraction (lane 2).

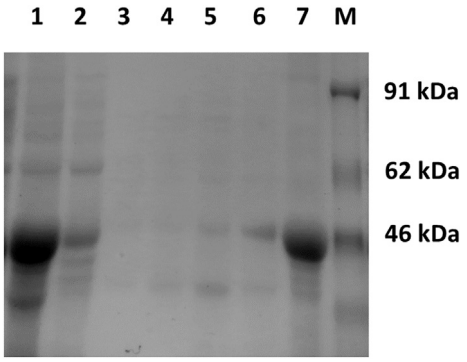

Figure 6. Protein purification using Ni-NTA agarose. Lane 1: denatured protein, lane 2–4: washing fractions, lane 5–6: elution fractions with pH 4.5 elution buffer, lane 7: elution fraction with TE elution buffer. M: Protein marker with indicated size.

3.5. Protein Purification

Purification using Ni-NTA agarose showed that target protein was successfully bound to Ni-NTA beads as can be seen that only small amount of the protein is found in flow through or washing fraction. Elution buffer with lower pH buffer could not release protein from bead. Elution of the target protein using Tris-EDTA buffer obtained results that most of target protein was successfully eluted and purified (Figure 6).

4. Discussion

KHV caused significant morbidity and mortality both in common carp and koi carp (Hedrick et al. 2000; Michel et al. 2010; Ilouze et al. 2011). The external signs of KHV infected fish are swollen and necrotic gill filaments, excessive mucus production or discolored patches on the skin (Hedrick et al. 2005). The pathological KHV signs in the gill rakers include increased subepithelial inflammation and congestion of blood vessels in the gill arch, accompanied by attenuation of the rakers' height. Severe pathological changes were noted in the kidney as a mild peritubular inflammatory infiltrate was evident at early infection and will be followed by a heavy interstitial inflammatory infiltrate, along with congestion of blood vessels (Ilouze et al. 2011). In this study, the carp from Magelang showing necrosis and white patches of gills was used. PCR using extracted DNA from those gills with Bercovier primer pairs confirmed that the fish was infected by KHV.

Analysis on KHV ORF2 with sample from Toba lake, North Sumatra (Murwantoko 2009), on KHV ORF124 with sample from Yogyakarta (Murwantoko et al. 2012), on marker I, marker II and thymidine kinase with sample from various areas in Indonesia (Sunarto et al. 2011) showed that all samples belonged to the KHV Asian lineage. In this study KHV ORF2 from Magelang, Central Java showed same homology with the KHV Asian and European genotypes. This result showed that ORF25 cannot be used to differentiate the lineage.

Vaccination is an important approach to control disease. Ronen et al. (2003) have proved that KHV can be developed as an efficient vaccine since using of attenuated virus on viral inoculation and challenge resulted in high levels of antibodies against the virus and very high anti-viral resistance. This result was also supported by Adkison et al. (2005) using an enzyme-linked immunosorbent assay, they detected the presence of anti-KHV antibodies in the serum of koi following either natural or experimental exposures to KHV. In order to reduce the possibility of the attenuated virus reverting to pathogenic, Perelberg et al. (2005) irradiated it and selected additional clones appropriate for vaccination. The results of their study suggest that a safe and efficient prophylactic vaccine can be developed by selecting an appropriate attenuated virus. However, Yuasa and Sano (2009) found that even though the attenuated vaccine is effective in preventing the disease, the risk of reversion to its pathogenic form is a potential threat.

Glycoprotein has been widely used for vaccine development against many viruses such as viral hemorrhagic septicemia (Lecocq-Xhonneux et al. 1994), hepatitis C virus (Stamataki et al. 2007), avian influenza virus (Yang et al. 2010). Herpesviruses employ the glycoproteins to enter cells, a process that involves fusion of the virion envelope with cellular membranes (Farnsworth et al. 2007). This KHV glycoprotein, ORF25 has been used for DNA vaccination against KHV and showed the protection (Nuryati et al. 2010; Nuswantoro et al. 2012; Zhou et al. 2014).

Beside the DNA vaccine, the recombinant proteins have been used widely in development of fish vaccine. Recombinant capsid protein of betanodavirus of nervous necrosis virus (Sommerset et al. 2005; Lin et al. 2007), coat protein of nervous necrosis virus (Kim et al. 2015), major coat protein of rock bream iridovirus (Shin et al. 2013), protein ORF093 of infectious spleen and kidney necrosis virus (ISKNV) (Li et al. 2015a), glycoprotein viral hemorrhagic septicemia (Lecocq-Xhonneux et al. 1994) has been developed as vaccines. The efficacy of vaccination methods should be tested; Sommerset et al. (2005) found that vaccination using recombinant capsid protein of nodavirus produced higher protection compared to recombinant DNA in turbot (*Scophthalmus maximus*). Similar pattern was recorded by Fu et al. (2015) using ORF086 of ISKNV on

Chinese perch (*Siniperca chuatsi*). In this study we expressed KHV ORF25 protein in *E. coli*.

The full length of KHV ORF25 from TUMSTI KHV strain composed by 1806 nt and encoded 601 amino acids. In this study the PCR amplicon length and sequencing result showed the DNA fragment length of only 1012 bp. The primer was designed to exclude the hydrophobic cluster area in order to achieve high protein production. As in high level expression, hydrophobic stretches in the polypeptide are present at high concentrations and available for interaction with similar regions. All these factors lead to protein instability and aggregation (Rosano and Ceccarelli 2014).

In this study the KHV ORF25 protein was successfully produced in *E. coli* BL21 under pET32a (+) system. The protein was expressed dominantly and it was insoluble. Use of T7 promoter system in that applied to the host will direct the host bacteria to produce target protein up to 50% from the total protein produced (Sambrook and Russel 2001). The protein expression of this study is better than the similar previous study by Murwantoko et al. (2012) which did not show the dominant protein expressed band. The KHV ORF25 protein size was approximately 45 kDa. That size comes from approximately 337 amino acid residues from KHV ORF25 of this study and 162 amino acid residues from plasmid pET32 at upstream of KHV ORF25 such as Trx-Tag, His-Tag and S-Tag proteins and 18 amino acid residues at downstream of KHV ORF25 up to stop codon of pET32a.

T-cell epitope prediction analysis of this KHV ORF25 protein showed that nine and eight epitopes were available based on IAd and Rothbard/Taylor patterns, respectively. B-cell epitope prediction showed this protein contains 3, 23, and 8 epitopes based on Emini surface accessibility, Karplus and Schulz flexibility and ElliPro scales, respectively. Many T-cell and B-cell epitopes were recognized by more than one pattern. Those results indicate that although this is partial protein of KHV ORF25, it is still predicted to have high immunogenicity.

Glycosylation is one of the most common posttranslational modifications of proteins. It has important roles for protein structure, stability and functions. *In vivo* the glycostructures influence pharmacokinetics and immunogenicity. It is well known that significant differences in glycosylation and glycostructures exist between recombinant proteins expressed in mammalian, yeast and insect cells. Different glycosylation in HEK and CHO mammalian cells has been observed by Croset et al. (2012). Glycoproteins are glycosylated with a bewilderingly heterogeneous array of complex *N*- and *O*-linked glycans, which are the product of the coordinated activity of enzymes resident in the endoplasmic reticulum and Golgi apparatus of the cell (Brooks 2004). Since in this study protein expression was on bacteria which do not have endoplasmic reticulum and Golgi apparatus, the glycosylation of the protein was expected to be very limited. To prove whether the lack of glycosylation affect immunogenicity, the *in vivo* immunogenicity and protection tests should be addressed.

Acknowledgments

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Conflict of interest

None.

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